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Identification of 69-kd and 100-kd forms of 2-5A synthetase in interferon-treated human cells by specific monoclonal antibodies

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Recently, the existence of 40-kd and 46-kd 2-5A synthetases in interferon-treated cells has been confirmed by cloning and characterization of cDNA corresponding to these small size enzymes. By the use of specific monoclonal antibodies, we describe here two forms of high mol. wt 2-5A synthetases of 69 and 100 kd in human cells. The monoclonal antibodies immunoprecipitate either a 69- or a 100-kd 2-5A synthetase. These purified 2-5A synthetases in immune complex preparations are active, i.e. addition of poly(I).poly(C) and ATP results in the synthesis of 2-5A. Both 2-5A synthetases are composed of several subspecies with similar isoelectric points in the range of 7-8 but have different subcellular localizations: 100-kd synthetase is recovered from the microsomal pellet whereas 69-kd synthetase is found to be associated with cell membranes as well as with the microsomal pellet. Different types of interferon-treated human cells express both or either forms of these enzymes. The 69- and 100-kd 2-5A synthetases were also identified by electrophoretic transfer immunoblot analysis using rabbit polyclonal antibodies against a synthetic peptide common on both 46- and 40-kd 2-5A synthetases. These results indicate that small and large size isozymes share a common peptide sequence.

Key words: interferon-induced proteins/2-5A synthetase/monoclonal antibodies/double-stranded RNA

Introduction

Two double-stranded (ds)RNA-dependent enzymes, a specific protein kinase and a 2-5A synthetase, are involved in the mechanism of action of interferon (see Hovanessian, 1985 for references). The protein kinase is a 68-kd protein (p68 kinase) which becomes autophosphorylated in the presence of dsRNA (Hovanessian and Galabru, 1986). This phosphorylated p68 kinase can then catalyse the phosphorylation of the α-subunit of protein synthesis initiation factor eIF2, thus mediating inhibition of the initiation of protein synthesis (Farrel *et al.*, 1977; Safer, 1983). The 2-5A synthetase is responsible for the synthesis of 2'-5' linked oligomers of adenosine, pppA(2'p5'A)$_n$ where n is 2 or more (Kerr and Brown, 1978). For convenience, these mixtures of oligonucleotides are referred to as 2-5A and the enzyme which synthesizes it is referred to as 2-5A synthetase. The 2-5A synthetase binds to poly(I).poly(C)-Sepharose and could be used in this form to synthesize 2-5A in the presence of ATP (Hovanessian *et al.*, 1977; Hovanessian and Kerr, 1979). The function of 2-5A is to activate a latent endonuclease responsible for degradation of viral and cellular RNAs (Baglioni *et al.*, 1978; Clemens and Williams, 1978; Hovanessian *et al.*, 1979).

The presence of 2-5A synthetase by its *in vitro* activity has been reported in the cytoplasm and in nuclei of different types of cells (Nilsen *et al.*, 1982; Smekens-Etienne *et al.*, 1983; St Laurent *et al.*, 1983; Schweiger and Kostka, 1984; Shimizu and Sokawa, 1983). St Laurent *et al.* (1983) have isolated two mRNAs coding for two different 2-5A synthetases of 20-30 kd and 85-100 kd, the larger enzyme being present in the cytoplasm and the smaller one in the nuclei. Recently, the cloning and characterization of cDNA corresponding to two small mol. wt 2-5A synthetases have been carried out successfully by several groups (Merlin *et al.*, 1983; Chebath *et al.*, 1985; Benech *et al.*, 1985a,b; Saunders *et al.*, 1985a,b; Wathelet *et al.*, 1986). These studies have indicated the existence of 40- and 46-kd 2-5A synthetases which are products of 1.6- and 1.8-kb mRNAs, respectively (Chebath *et al.*, 1985). The 40- and 46-kd synthetases are derived from the same gene. They are identical in their first 346 residues but are different at their C-terminal ends (Benech *et al.*, 1985b).

Partial purification of 2-5A synthetase by conventional techniques has suggested the presence of different forms of this enzyme. For example, Revel *et al.* (1982) have reported the existence of 30-40 kd and 60-80 kd enzymes in human SV80 and HeLa cells. In human Namalwa cells, they could detect only a 30-kd enzyme. A 100-kd 2-5A synthetase has been described in HeLa cells by several groups (Yang *et al.*, 1981; Wells *et al.*, 1984; Ilson *et al.*, 1986). Large and small size 2-5A synthetases have also been reported in mouse Ehrlich ascites tumor cells, in porcine cells and in avian erythrocytes (Dougherty *et al.*, 1980; St Laurent *et al.*, 1983; Shimizu *et al.*, 1984; Sokawa *et al.*, 1984). The relationship between the small and large forms of 2-5A synthetases is not yet clear. There is no doubt that the 40- and 46-kd enzymes are the products of the 2-5A synthetase gene described by several groups. Recently, Chebath *et al.* (1986) showed that antibodies against a synthetic peptide derived from the cDNA sequence of 40- and 46-kd enzymes recognize, by immunoblotting, four interferon-induced proteins of mol. wt 40 000, 46 000, 69 000 and 100 000 daltons. Partial purification of these proteins by sedimentation on glycerol gradients, DEAE-cellulose and CM-cellulose chromatography techniques resulted in the recovery of 2-5A synthetase activity in fractions containing each protein. We describe here the isolation of monoclonal antibodies directed against 2-5A synthetases of human origin. These antibodies immunoprecipitate either a 69- or a 100-kd protein, each with a characteristic activity of 2-5A synthetase. These results therefore confirm the existence of distinct 69- and 100-kd forms of 2-5A synthetases. These enzymes are present in different types of cells and are highly induced by interferon.

Results

Isolation and characterization of monoclonal antibodies against human 2-5A synthetase

The presence of antibodies in the serum of immunized mice and in hybridoma culture supernatants was detected by an immunoprecipitation assay using a buffer containing high concentrations of both salt and non-ionic detergent (buffer I.P.). Briefly, extracts

from interferon-treated Daudi cells were incubated with the antibody preparation and protein A−Sepharose. Immune complexes bound to the solid support were first washed with buffer I.P. and then incubated with [^{3}H]ATP and poly(I).poly(C) to synthesize 2-5A. The dsRNA was required for the activation of 2-5A synthetase in immune complex preparations. The presence of antibody, therefore, was detected indirectly by the activity of 2-5A synthetase.

Five mice were injected five times with a partially purified preparation of 2-5A synthetase. Such preparations consisted of poly(I).poly(C)−Sepharose bound proteins from interferon-treated cells (Hovanessian and Kerr, 1979). After the fourth immunization, three mice were found to produce specific antibodies detectable by the immunoprecipitation technique (Materials and methods). Hybridoma cell lines were obtained by fusing splenocytes from one of the immune mice with X68/Ag8 myeloma cells. Culture supernatants were tested for the presence of specific antibodies by the immunoprecipitation assay for the 2-5A synthetase activity. Six hybridomas were found to produce antibodies against 2-5A synthetase (Table I). The selected hybridomas and the serum of the sacrificed mouse were further studied by immunoprecipitation of [^{35}S]cysteine-labeled proteins from control and interferon-treated cells (Figure 1). Serum from normal immunized mouse did not precipitate [^{35}S]cysteine-labeled proteins (data not shown). On the other hand, serum from the immune-positive mouse was found to immunoprecipitate several proteins of mol. wt 35 000−200 000 daltons. Among these proteins, two proteins of mol. wt 85 000 and 200 000 were found to be reduced whereas at least six proteins of mol. wt 35 000, 69 000, 75 000, 100 000, 120 000 and 150 000 were enhanced in interferon-treated cells. Supernatants of the six antibody-positive hybridoma cultures resulted in the immunoprecipitation of either a 69 000- or 100 00-dalton protein, both of which were significantly induced by interferon (Figure 1, lanes 56, 44, 17, 25, 26, 28). Such immune complex preparations from the six hybridoma culture supernatants were also active for the synthesis of 2-5A (Table I). In view of this and the fact that no other protein was detectable in these immune complex preparations, they will be referred to as 69- and 100-kd 2-5A synthetases. It is interesting here to note that comparable amounts of [^{35}S]cysteine-labeled 69-kd protein were immunoprecipitated by the antibodies of hybridomas 44 and 56 (Figure 1). However, similar immune complex preparations showed >8-fold higher activity to synthesize 2-5A in the preparation using antibody of hybridoma 56 than that of hybridoma 44 (Table I). A similar result was obtained using antibodies precipitating 100-kd 2-5A synthetase, i.e. immune complex preparations from hybridomas 17, 25, 26 and 28; each one precipitated comparable amounts of 100-kd protein but manifested different levels of 2-5A synthetase activity (Figure 1 and Table I). Therefore, some of the antibodies cause partial inhibition of enzyme activity, indicating that different epitopes on 69- and 100-kd proteins are recognized by the two groups of antibodies (44 and 56; 17, 25, 26 and 28).

The six antibody-positive hybridomas were further subcloned and screened for antibody production (as above). During this procedure, hybridoma 26 lost its capacity to produce antibodies. Several subclones (17/17, 25/11, 28/18, 44/11 and 56/3) were selected and the antibody produced by each clone was characterized (Table II). Clones 28/18 and 44/11 were producers of IgG1 whereas clones 17/17, 25/11 and 56/3 were producers of IgG2a class antibodies. Monoclonal antibodies produced by clones 56/3 and 25/11 were used in the experiments discussed here to characterize 69- and 100-kd 2-5A synthetases, respectively. All the different monoclonal antibodies did not recognize denatured forms of 69- and 100-kd proteins.

Table I. 2-5A Synthetase activity in immune complex preparations from hybridoma cultures

Antibody sample		2-5A Synthetase activity ([^{3}H]c.p.m./assay)
–		3261
Control serum		3155
Immune serum		82 463
Culture medium		3577
Hybridoma culture	56	388 011
	44	45 609
	17	106 409
	25	165 468
	26	86 608
	28	79 752

Immune complex preparations were obtained by incubation of extracts from interferon-treated Daudi cells with antibody samples: serum (3 μl) from an unimmunized mouse; serum (3 μl) from the immune mouse which was sacrificed for the preparation of hybridomas; supernatants (200 μl) of hybridoma cell cultures. The presence of 2-5A synthetase was detected by its capacity to synthesize 2-5A after an overnight incubation ([^{3}H]c.p.m./assay). Input [^{3}H]ATP (5 mM) was 712 566 c.p.m. whereas background c.p.m. was 3500.

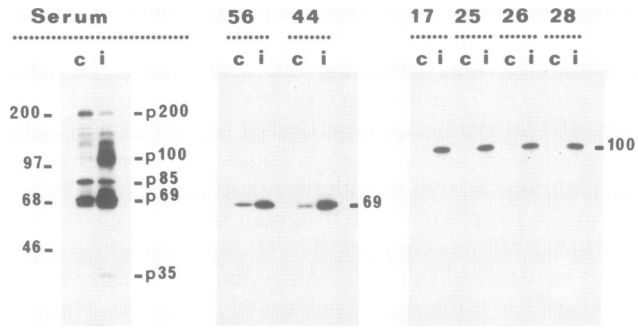

Fig. 1. Immunoprecipitation of [^{35}S]cysteine-labeled proteins from control and interferon-treated Daudi cells. [^{35}S]cysteine-labeled extracts from control **(lanes c)** and interferon-treated **(lanes i)** Daudi cells immunoprecipitated using serum (5 μl) from the immune mouse and supernatant (200 μl) from hybridoma cultures numbers 56, 44, 17, 25, 26, 28. Immune complex preparations recovered by protein A−Sepharose were washed in buffer I.P. and analysed by polyacrylamide (10%) gel electrophoresis. A fluorograph of the gel is shown. On the left are the positions of protein mol. wt markers: myosin, 200 000; phosphorylase B, 97 000; bovine serum albumin, 68 000; ovalbumin, 46 000. On the left of Serum section are indicated the proteins modified by interferon: p200, p100, p85, p69 and p35. Antibodies from hybridoma cultures 56 and 44 specifically immunoprecipitate a protein of mol. wt 69 000. Antibodies from hybridoma cultures 17, 25, 26 and 28 immunoprecipitate a protein of mol. wt 100 000.

The other two immune mice were sacrificed after the seventh immunization and hybridoma cell lines were prepared as above. Five hybridomas obtained from one of these mice were producers of anti-2-5A synthetase antibodies. These hybridomas were subcloned and were all found to be producers of antibodies against 100-kd 2-5A synthetase. These monoclonal antibodies (114/25, 171/2 and 185/45) were of IgG1 and IgG2a class antibodies (Table II).

Identification of 69- and 100-kd 2-5A synthetases by rabbit anti-peptide B

The identity of 69- and 100-kd 2-5A synthetases was confirmed by immunoblotting using rabbit serum containing antibodies

Table II. Characterization of the different monoclonal antibody-producing subclones

Subclone (ascitic fluid)	IgG class	Immunoprecipitated protein (kd)	2-mercaptoethanol activity ([^{3}H]c.p.m.)
I: 17/17	IgG2a	100	215 668
25/11	IgG2a	100	236 125
28/18	IgG1	100	120 495
44/11	IgG1	69	105 375
56/3	IgG2a	69	412 011
II: 114/25	IgG1	100	127 547
171/2	IgG2a	100	245 283
185/45	IgG2a	100	228 661

Two sets of subclones obtained from the I and II fusion experiments (mice I and II) were injected into pristane-primed mice for the production of ascitic fluid. 5 μl aliquots of these ascitic fluids were analysed for the immunoprecipitation of [^{35}S]cysteine-labeled proteins and assayed for 2-5A synthetase activity (as in Figure 1 and Table I). Isotyping of monoclonal antibodies was determined by Ouchterlony's immunodiffusion test.

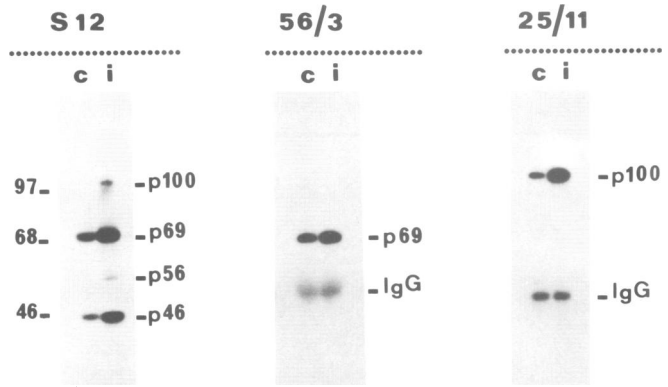

Fig. 2. Identification of 69-kd and 100-kd proteins by immunoblotting using rabbit anti-peptide B serum. Western blot analysis was carried out using a rabbit serum containing antibodies (anti-peptide B) against a synthetic peptide derived from the cDNA sequence of 40- and 46-kd 2-5A synthetases (Chebath *et al.*, 1986). Section S12: crude extracts from control (c) and interferon-treated (i) Daudi cells. Section 56/3 and 25/11: immune complex preparations using crude extracts and monoclonal antibodies 56/3 and 25/11. All the samples were analysed by polyacrylamide (10%) gel electrophoresis and processed for Western blot analysis. An autoradiograph is shown. Four proteins were recognized in the S12 sample: p100, p69, p56 and p46. In the immune complex preparations, a protein of mol. wt 55 000 was detectable in addition to 69- and 100-kd proteins. This protein is the heavy chain of the monoclonal antibodies 56/3 or 25/11 which was recognized by ^{125}I-labeled protein A.

against a synthetic peptide derived from the cDNA sequence of 40- and 46-kd 2-5A synthetases. These antibodies, known as anti-peptide B, were raised in a rabbit immunized with a synthetic peptide comprising amino acids 284–303 in the part common to 40- and 46-kd enzymes (Chebath *et al.*, 1986). Anti-peptide B recognizes several proteins in crude extracts of human cells by electrophoretic transfer immunoblot analysis (Western blot). Figure 2 (section S12) shows the results of such Western blot analysis in extracts from control and interferon-treated Daudi cells. Three major proteins of mol. wt 46 000, 69 000 and 100 000 are detectable at enhanced levels in interferon-treated cells. Daudi cells do not have 40-kd 2-5A synthetase since they do not express the 1.6-kb mRNA. The 46-kd 2-5A synthetase is the product of a 1.8-kb mRNA (Chebath *et al.*, 1986). The 69- and 100-kd proteins, as they have been previously reported,

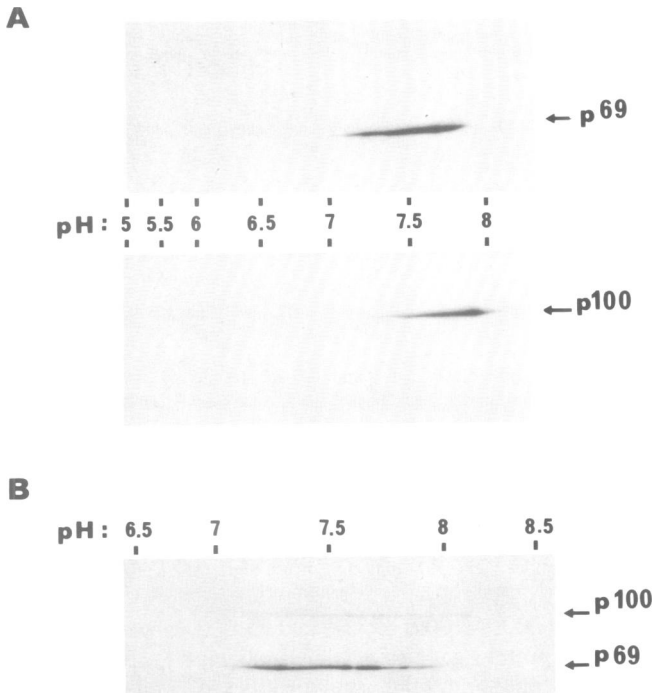

Fig. 3. Two-dimensional gel electrophoretic analysis of 69- and 100-kd proteins. Purified [^{35}S]cysteine-labeled 69- and 100-kd proteins (prepared by immunoprecipitation using monoclonal antibodies) were analysed by two-dimensional gel electrophoresis as described previously (O'Farrel, 1975; Krust *et al.*, 1982). The pH gradient obtained by isoelectric focusing (1st dimension) was between 5 and 7.8 in **A** and 6.5 and 8.5 in **B**. In **B**, the two proteins were mixed and analysed together. A fluorograph is shown. The concentration of ampholine used in **A** was 2%, pH range 3–10 while in **B** it was 1%, pH range 5–8, 0.5%, pH range 6.5–9 and 1%, pH range 8–10.

manifest 2-5A synthetase activity (Tables I and II, Figure 1). Anti-peptide B identifies 69- and 100-kd 2-5A synthetases in the immune complex samples prepared using monoclonal antibodies 56/3 and 25/11, respectively (Figure 2, sections 56/3 and 25/11).

In addition to 46-, 69- and 100-kd proteins, anti-peptide B recognizes a 56-kd protein in crude Daudi extracts. This protein is induced by interferon but its identity is not known. It might be another 2-5A synthetase or a different protein which shares a common peptide sequence with 46-, 69- and 100-kd 2-5A synthetases.

Characterization of 69- and 100-kd 2-5A synthetases

[^{35}S]cysteine-labeled 69- and 100-kd proteins were analysed by two-dimensional gel electrophoresis. Figure 3A shows that both of these proteins are composed of several subspecies with similar isoelectric points (pIs). The pIs of 69-kd protein are in the range of 7–7.8 whereas 100-kd protein is slightly more basic with pIs in the range of 7.4–8.1. Purified 69- and 100-kd proteins were mixed together and analysed by two-dimensional gel electrophoresis using a wider pH gradient in the first dimension. In this gradient, both proteins were resolved as a streak in the pH region between 7 and 8.2 (Figure 3B). Once again 100-kd protein was slightly more basic than 69-kd protein.

[^{35}S]cysteine-labeled 69- and 100-kd proteins were further characterized according to their susceptibility to partial digestion by *Staphylococcus aureus* V8 protease (Figure 4). Partial proteolysis of 69-kd protein gave rise to four major polypeptides of

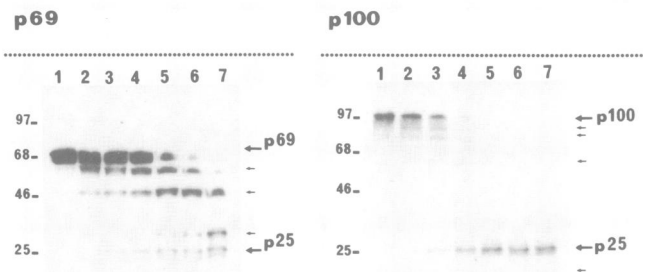

Fig. 4. Limited proteolysis of 69- and 100-kd proteins by *S. aureus* V8 protease. [^{35}S]cysteine-labeled 69- and 100-kd proteins were analysed by limited proteolysis at different concentrations of *S. aureus* V8 protease: 0 **(lanes 1)**, 0.1 **(lanes 2)**, 0.5 **(lanes 3)**, 1 **(lanes 4)**, 2 **(lanes 5)**, 10 **(lanes 6)** and 20 **(lanes 7)** μg per slot (Krust *et al.*, 1984). Fluorographs of the gel (12.5%) are shown. The positions of 69- (p69) and 100-kd (p100) proteins are indicated on the right side of each gel. The p25 seems to be a common digestion product of 69- and and 100-kd proteins. The small arrows indicate other polypeptides yielded by digestion of 69- and 100-kd proteins.

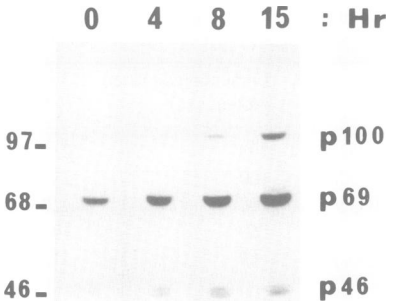

Fig. 5. Kinetics of enhancement of 46-, 69- and 100-kd proteins in interferon-treated Daudi cells. Extracts from interferon-treated Daudi cells at 0, 4, 8 and 15 h after addition of interferon were analyzed by Western blot using rabbit serum containing anti-peptide B antibodies. The binding of rabbit IgG to 46-, 69- and 100-kd proteins was revealed by ^{125}I-labeled protein A. An autoradiograph is shown. In this experiment, 56-kd protein was not detected (see Figure 2).

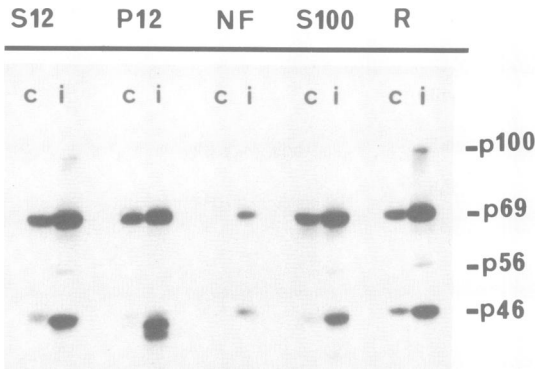

Fig. 6. Immunoblot analysis of different subcellular fractions using anti-peptide B serum. Subcellular fractions from control **(lanes c)** and interferon-treated *(lanes i)* Daudi cells were prepared as described in Materials and methods. S12: post-mitochondrial supernatant; P12, mitochondrial pellet; NF: nuclear fraction; S100: post-rough microsomal pellet. Extracts corresponding to material from 2.5 × 10^5 cells were analyzed by polyacrylamide (10%) gel electrophoresis and processed for Western blotting using rabbit serum containing anti-peptide B antibodies (as in Figure 5). An autoradiograph is shown. The positions of 100-, 69-, 56- and 46-kd proteins are indicated on the right.

mol. wt 60 000, 46 000, 32 000 and 25 000, whereas digestion of 100-kd protein gave rise to one major polypeptide of mol. wt 25 000 and four minor polypeptides of mol. wt 85 000, 75 000, 60 000 and 20 000. Among these polypeptides, the one of mol. wt 25 000 (p25) was found to be a common partial digestion product of 69- and 100-kd proteins. At different concentrations of *S. aureus* V8 protease, p25 was detectable in both cases and became accumulated at higher concentrations of the protease (Figure 4, lanes 2−7). It might be, therefore, that this p25 fragment from 69- and 100-kd proteins is a common polypeptide between these two 2-5A synthetase species.

The anti-peptide B which was raised against a peptide of 46-kd 2-5A synthetase recognizes 69- and 100-kd proteins as well as 46-kd protein by Western blot analysis (Figure 2). In view of this, and the observations discussed in Figures 3 and 4, it remained possible that 69- and 100-kd proteins are post-translationally modified species of 46-kd protein. If this were the case then the anti-peptide B would have identified proteins of lower mol. wt than 69 000 and 100 000 as hypothetical precursors of 69- and 100-kd proteins. In Figure 5, anti-peptide B was employed in a Western blot experiment using crude extracts from interferon-treated Daudi cells, at different times after addition of interferon. It is interesting to note that the level of 46-, 69- and 100-kd proteins are enhanced in response to treatment with interferon. The kinetics of enhancement for each protein indicates that 4 h after addition of interferon the three proteins (46, 69 and 100 kd) are enhanced with increased levels at 15 h. Anti-peptide B could not identify other proteins of intermediate mol. wt between 46 000 and 69 000−100 000 (Figure 5).

Distribution of different species of 2-5A synthetase in subcellular fractions

In these experiments, we investigated the distribution of 46-, 69- and 100-kd 2-5A synthetases in control and interferon-treated Daudi cells by Western blot analysis using anti-peptide B serum (Figure 6). Control and interferon-treated Daudi cells were homogenized in low salt buffer and processed for the preparation of nuclear fraction (NF), post-mitochondrial supernatant (S12), mitochondrial pellet (P12), post-rough microsomal supernatant (S100) and rough microsomal pellet (RMP) consisting of endoplastic reticulum and ribosomes. The 46- and 69-kd proteins which were present in crude extracts were isolated with the different subcellular fractions, P12, NF, S100 and RMP. On the other hand, the 100-kd protein was only detected in the RMP fraction (Figure 6). The 56-kd protein of unknown function which is recognized by anti-peptide B was found in both S100 and RMP fractions. The 46-kd protein in the P12 fraction was identified as a doublet. This might be due to the lower concentration of proteins in the P12 fraction compared with S100 and RMP fractions. The 69-kd protein might also be a doublet since it is revealed as a wide band by Western blot analysis, Furthermore, [^{35}S]cysteine-labeled 69-kd protein migrates as a wide band during polyacrylamide gel electrophoretic analysis and in some experiments it could be observed as a doublet (Figure 4). The 69- and 100-kd proteins in the different subcellular fractions manifest 2-5A synthetase activity after purification by the monoclonal antibodies 56/3 and 25/11, respectively (Table III). These

Table III. 69-kd and 100-kd 2-5A synthetase activities in different subcellular fractions from control and interferon-treated Daudi cells

Subcellular fraction	Cells	2-5A Synthetase activity (nmol AMP/h)	
		69-kd	100-kd
S12	C	21.7	6.3
	IFN	84.9	61.4
P12	C	11.2	<0.1
	IFN	31.4	<0.1
NF	C	0.5	<0.1
	IFN	6.3	<0.1
S100	C	8.7	0.7
	IFN	30.4	10.4
RMP	C	7.2	3.8
	IFN	34.3	58.2

Subcellular fractions from control (C) and interferon-treated (IFN) Daudi cells were prepared as described in Materials and methods. S12: post-mitochondrial supernatant; P12: mitochondrial pellet; NF: nuclear fraction; S100: post-rough microsomal supernatant; RMP: rough microsomal pellet. Aliquots from each fraction were immunoprecipitated (equivalent to that from 1×10^7 cells) using monoclonal antibodies 56/3 (to isolate 69-kd protein) or 25/11 (to isolate 100-kd protein) and the immune complex preparations were assayed for synthesis of 2-5A by overnight incubation in a reaction mixture at pH 6.5 or 7.5 according to the specific monoclonal antibody. The results are expressed in nmol of AMP incorporated per hour.

Table IV. Extraction of 69-kd and 100-kd 2-5A synthetases associated with the rough microsomal pellet

Extraction	Fraction	% 2-5A synthetase activity	
		69-kd	100-kd
Buffer RMP	S100	5.1	4.3
	Pellet	95.3	86.6
1% Triton	S100	21.4	2.1
	Pellet	65.5	90.7
0.5 M KCl	S100	6.4	88.4
	Pellet	90.5	10.5
1% Triton + 0.5 M KCl	S100	87.6	92.1
	Pellet	2.8	8.9

The RMP (from 10^9 interferon-treated Daudi cells) prepared as in Figure 6 and Table III was suspended in buffer RMP (10 mM Tris-HCl pH 7.6, 100 mM sucrose, 50 mM KCl, 1 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 1000 units/ml aprotinin and 0.2 mM PMSF) before dividing into four equal aliquots. These samples were then incubated at 4°C for 30 min in buffer RMP in the absence and presence of 1% Triton X-100 and 0.5 M KCl or both 1% Triton X-100 and 0.5 M KCl. After centrifugation at 100 000 *g* (2 h, 4°C), the supernatant (S100) and the pellet of each suspension were assayed for 2-5A synthetase activities of 69-kd and 100-kd proteins. The results are expressed as % activity recovered in the S100 and pellet after the second centrifugation compared with the activity of each enzyme in the initial RMP preparation.

results indicate that 69- and 100-kd proteins associated with the different cellular fractions are active. The membrane-rich mitochondrial pellet (P12) fraction was further separated by centrifugation on a Percoll gradient. The plasma membranes recovered at a buoyant density of 1.016 and 1.048 contained both 46- and 69-kd proteins (data not shown).

The 69- and 100-kd proteins are tightly associated with the rough microsomal pellet. The 69-kd protein is slightly solubilized by 1% Triton X-100 and it becomes almost completely solubilized by an extraction buffer containing 1% Triton X-100 and 0.5 M KCl. On the other hand, 100-kd protein is efficiently recovered by 0.5 M KCl whereas 1% Triton has no effect on its solubilization (Table IV). These results indicate that the interactions of 69- and 100-kd 2-5A synthetases with the rough microsomal pellet are different.

Table V. Activities of 69- and 100-kd 2-5A synthetases in different types of cells

Cells: control or interferon-treated		Activity of 2-5A synthetase (nmol AMP/10^7 cells/h)	
		69-kd	100-kd
BJAB:	C	1.5	6.8
	IFN	62.7	52.9
HeLa:	C	<0.1	20.9
	IFN	7.1	56.5
UAC:	C	0.1	2.5
	IFN	0.9	70.4
T98G:	C	2.6	1.5
	IFN	45.3	5.9
L-929:	C	<0.1	<0.1
	IFN	<0.1	<0.1

Extracts from control (C) and interferon-treated (IFN) cells were assayed for 2-5A synthetase activities of 69-kd and 100-kd proteins. The results are presented in nmol of AMP incorporated per hour per enzyme preparation purified from 10^7 cells. Human cells were treated with human α-interferon, 500 units/ml for 18 h. Mouse L-929 cells were treated with mouse $\alpha + \beta$ interferon, 500 units/ml for 18 h (Galabru *et al.*, 1985).

Induction of 69- and 100-kd 2-5A synthetases in different types of human cells

Different types of human cells, BJAB (Burkitt's lymphoma cell line), HeLa, UAC (human amniotic U cells) and T98G (human glioblastoma cells) were investigated for the presence and induction of 69- and 100-kd synthetases in control and α-interferon-treated cells. These enzymes in crude extracts were purified by immunoprecipitation using monoclonal antibodies (56/3 and 25/11) and assayed for 2-5A synthetase activity (Table V). In contrast to control Daudi cells, very little 69-kd 2-5A synthetase was detectable in different types of human control cells studied here. A significant induction by interferon was observed in BJAB and T98G cells compared with HeLa cells which showed a much lower induction. A considerable activity of 100-kd 2-5A synthetase was detected in control BJAB, HeLa and UAC cells which also responded to interferon treatment by enhanced levels of this enzyme. T98G cells manifested a slight 100-kd 2-5A synthetase activity in both control and interferon-treated cells. No 2-5A synthetase activity was recovered in immune complex preparations from control and interferon-treated murine L-929 cells, thus suggesting that the monoclonal antibodies are specific of enzymes of human origin.

These results were further confirmed by immunoblot analysis using anti-peptide B serum (Figure 7). In addition to 69- and 100-kd 2-5A synthetases, this technique identifies also the small size 2-5A synthetases: 46 kd in BJAB cells; 40 kd in HeLa, UAC and T98G cells. Such differences in the molecular mass of the small size 2-5A synthetases correspond to the expression of 1.6-kb and 1.8-kb mRNA species in these human cell lines (Benech *et al.*, 1985;. Saunders *et al.*, 1985; Wathelet *et al.*, 1986). The results obtained by the immunoblot analysis indicate that the distribution and the level of each species of 2-5A synthetase varies between cell lines: BJAB cells express three major synthetases of 46, 69 and 100 kd; HeLa and UAC cells express mainly 100-kd 2-5A synthetase whereas 69- and 40-kd 2-5A synthetases

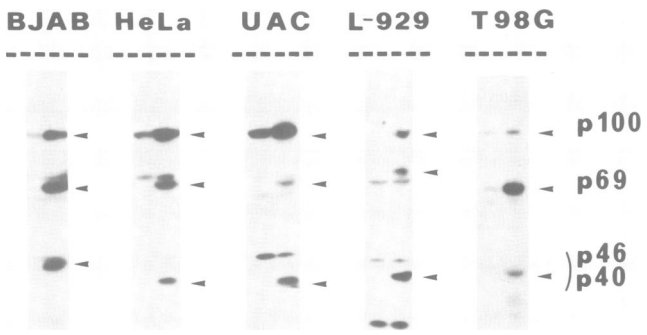

Fig. 7. Induction of different species of 2-5A synthetase in human cells treated with interferon. Extracts (from 2.5 × 10^6 cells) from control **(lanes c)** and interferon-treated **(lanes i)** cells were analysed by polyacrylamide gel electrophoresis and processed for Western blotting using rabbit serum containing anti-peptide B antibodies. The samples were the same as in Table V. An autoradiograph is shown. The position of 100-, 69, 46- and 40-kd proteins are indicated by the arrowheads.

are expressed at lower levels; T98G cells express high levels of 69-kd 2-5A synthetase and low levels of 40- and 100-kd 2-5A synthetases (Figure 7). Anti-peptide B serum can recognize several interferon-induced proteins in L-929 cells of 40, 75 and 100 kd (Figure 7). These proteins might correspond to different 2-5A synthetase species in this murine cell line.

Discussion

The cDNAs of two small size 2-5A synthetases of 40 and 46 kd have been cloned and sequenced (Benech *et al.*, 1985; Saunders *et al.*, 1985a; Wathelet *et al.*, 1986). These human proteins are coded by 1.6- and 1.8-kb mRNAs. The 1.6-kb RNA codes for a protein (40 kd) of 364 amino acids while the 1.8-kb mRNA codes for a protein (46 kd) of 400 residues, both of these proteins are identical in their first 346 residues (Benech *et al.*, 1985a,b). In addition to these small size enzymes, several reports in the literature have suggested the presence of larger size species of 2-5A synthetase in different types of human cells (Revel *et al.*, 1982; Yang *et al.*, 1981; Wells *et al.*, 1984; Ilson *et al.*, 1986; Chebath *et al.*, 1986). The results presented here show and confirm the existence of large size forms of 2-5A synthetase in different types of cells. With the help of specific monoclonal antibodies, here we show the characterization of 69- and 100-kd 2-5A synthetases.

Several hybridoma subclones producing anti-2-5A synthetase antibodies were isolated. These antibodies were selected by their capacity to immunoprecipitate the 2-5A synthetase without interfering with its activity. The choice of buffer used during this immunoprecipitation reaction is very critical because it should not inactivate the enzyme while in the meantime being efficient in washing away all proteins associated unspecifically with the immune complex bound to protein A−Sepharose. These requirements were fulfilled satisfactorily by buffer I.P. which contains 0.4 M NaCl and 1% Triton X-100. Thus we could isolate monoclonal antibodies which immunoprecipitated specifically one protein, either 69 or 100 kd. Each one of these proteins shows 2-5A synthetase activity dependent on dsRNA, indicating that they are large size 2-5A synthetases. No neutralizing antibodies were detectable even in the serum of immune mouse. It might be possible that the catalytic and dsRNA binding sites of the different 2-5A synthetases are not immunogenic since these sites are homologous in the human and murine enzymes. Mice were immunized with a 2-5A synthetase preparation containing the three species of enzymes: 46, 69 and 100 kd. However, no antibodies were detectable against 46-kd protein in the serum of immune mice. The strong conservation of murine and human small size 2-5A synthetase gene might account for the poor immunogenicity of human 46-kd 2-5A synthetase in mice (Saunders *et al.*, 1985b). The monoclonal antibodies 44/11 and 56/3 immunoprecipitate 69-kd 2-5A synthetase whereas monoclonal antibodies 17/17, 25/11, 28/18, 114/25, 171/2 and 185/45 all immunoprecipitate 100-kd 2-5A synthetase. The observation that some of these antibodies slightly modify the activity of 2-5A synthetase suggests that they are directed against different immune determinants on 69- and 100-kd proteins.

The degree of homology at the RNA level between small size (40 and 46 kd) and large size (69 and 100 kd) 2-5A synthetases is not known. As yet mRNAs encoding for human 69- and 100-kd 2-5A synthetases have not been identified. The 1.6- and 1.8-kb mRNAs of 40- and 46-kd 2-5A synthetases differ slightly in their C-terminal ends. The cDNAs of these small size 2-5A synthetases hybridize mainly to 1.6- or 1.8-kb RNAs according to the cell line studied: 1.6 kb in SV80, HeLa, UAC and T98G cells; 1.8 kb in SV80, Wish, Namalwa and Daudi cells (Benech *et al.*, 1985a; Saunders *et al.*, 1985a; Wathelet *et al.*, 1986). In some cell lines, such as HeLa and SV80 cells, 2.5−2.7 kb and 3.5−3.6 kb RNAs are also recognized by Northern blot analysis using the cDNA of the small size 2-5A synthetases (Benech *et al.*, 1985a,b; Saunders, 1985a). These large 2.5−2.7 and 3.5−3.6 kb RNAs are adequate in length to encode 69- and 100-kd 2-5A synthetases.

However, such RNA species are not systematically detectable in all interferon-treated cells which synthesize either or both of 69- and 100-kd 2-5A synthetases (Figures 6 and 7, Tables IV and V; Chebath *et al.*, 1986; Benech *et al.*, 1985a; Wathelet *et al.*, 1986). In SV80 cells, there seems to be a good correlation between the expression of 1.6-, 1.8-, 2.7- and 3.6-kb RNA species and the presence of four different 2-5A synthetases of 40, 46, 69 and 100 kd. Similarly, 1.6-, 2.7- and 3.6-kb RNA species correspond with the presence of 40-, 69- and 100-kd 2-5A synthetases in interferon-treated HeLa cells. On the other hand, only the 1.8-kb RNA is detectable in lymphoblastoid cells which synthesize 69- and 100-kd 2-5A synthetases in addition to the 46-kd enzyme. T98G and UAC cells express mainly the 1.6-kb RNA, but the main 2-5A synthetase formed in these cells is not the 46-kd enzyme; T98G cells express mainly the 69-kd enzyme whereas UAC cells express mainly the 100-kd enzyme. This discordance between detection of large size RNA species using the cloned cDNA as probes and the expression of large size forms of 2-5A synthetase might suggest that these large RNAs do not encode 69- and 100-kd proteins. The 2.7- and 3.6-kb RNA species, therefore, could be precursors of 1.6- and 1.8-kb RNAs (Benech *et al.*, 1985a). Consequently, the large 2-5A synthetases could be encoded by different genes from the gene which has been cloned previously. The monoclonal antibodies described here should provide efficient means in studies concerning characterization of mRNAs encoding 69- and 100-kd 2-5A synthetases. Human chromosomes 11 and 12 have been suggested to encode 2-5A synthetase (Shulman *et al.*, 1984; Williams *et al.*, 1986). Using a cDNA probe, the small size 2-5A synthetases have been assigned to chromosome 12 (Williams *et al.*, 1986) whereas the evidence for chromosome 11 has been obtained using enzyme activity assay (Shulman *et al.*, 1984). Thus, chromosome 11 might encode either or both of the 69- and 100-kd enzymes. Isolation of mRNAs coding for 69- and 100-kd 2-5A synthetases

remains to be done and in this respect our monoclonal antibodies should prove to be of great use.

In mouse Ehrlich ascites cells, two interferon-induced mRNAs of 1.5 and 3.8 kb have been isolated (St Laurent *et al.*, 1984). These mRNAs apparently code 20–30 kd and 85–100 kd 2-5A synthetases. Interestingly, among interferon-induced proteins in mouse L-929 cells, there is a 85-kd protein which is identified by immunoblot analysis using rabbit anti-peptide B antibodies specific for human 2-5A synthetase.

Materials and methods

Reagents

[^{3}H]ATP, [^{35}S]cysteine and ^{125}I-labeled protein A (with Bolton and Hunter reagent) were supplied by Amersham. Protein A–Sepharose CL-4B was from Pharmacia. Actinomycin D was from Boehringer Mannheim. *S. aureus* V8 protease (EC 3.4.21.19) was purchased from Miles Laboratories (UK). Poly(I).poly(C)–Sepharose CL-4B was prepared as described (Hovanessian *et al.*, 1983). Human leucocyte (α) interferon (10^8 NIH units/mg of protein) was purified by immunoaffinity chromatography using monoclonal antibody specific for human α-interferon (Meurs *et al.*, 1983). Poly(I).poly(C) and poly(A).poly(U) were from P.L. Biochemicals; the sedimentation values ($S_{20,w}$) of these complexes were >12 and 10, respectively. Aprotinin was under the commercial name of Iniprol (Laboratoire Choay, Paris, France). Rabbit sera containing anti-peptide B antibodies were prepared as described (Chebath *et al.*, 1986). Peptide B refers to a synthetic peptide comprising amino acids 284–303 in the part common to the small size 2-5A synthetases (40 and 46 kd).

Buffers

Homogenization buffer. 10 mM Tris-HCl (pH 7.6), 10 mM KCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1000 units/ml aprotinin and 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

Lysis buffer. Homogenization buffer containing 2% Triton X-100.

Immunoprecipitation buffer (buffer I.P.). 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 400 mM NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, 0.2 mM PMSF, 100 units/ml aprotinin and 20% glycerol (v/v).

Buffer A. 10 mM 4-(2-hydroxyethyl-)-1-piperazineethanesulfonic acid (Hepes), pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 7 mM 2-mercaptoethanol and 20% glycerol (v/v).

Buffer B. 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 units/ml aprotinin and 20% glycerol (v/v).

Cell cultures and extracts

Human Daudi and BJAB cells were grown in suspension in RPMI 1640 medium (Grand Island Biological Company, USA) containing 10^{-5} M 2-mercaptoethanol and 10% fetal calf serum (FCS). Human amniotic U cells (UAC) were grown in minimum essential medium containing 10% FCS. Human glioblastoma (T89G), HeLa (MRL2) and mouse L-292 cells were grown as monolayers in Dulbecco's modified medium containing 10% FCS. For the preparation of cell extracts, cells were washed with phosphate-buffered saline (PBS) before first suspension in homogenization buffer (1 packed-cell volume) and then extracting with lysis buffer (1 packed-cell volume). Cell extracts were then diluted with buffer I.P. and centrifuged at 1500 *g* and at 12 000 *g*. The supernatants after 12 000 *g* centrifugation were stored at −80°C.

Immunization of mice

Five BALB/c mice were injected i.p., five times at 10 day intervals, with the 2-5A synthetase attached to poly(I).poly(C)–Sepharose (200 μl). Material in each injection was equivalent to that from 5×10^6 Daudi cells treated with interferon. Poly(I).poly(C)–Sepharose-bound 2-5A synthetase preparations were washed extensively in buffer B before final wash in PBS containing 1000 units/ml aprotinin. Mice were injected with such preparations along with poly(A).poly(U) (200 μg/mouse). Three mice were found to produce antibodies after five injections. These antibodies were tested by an immunoprecipitation assay in which the 2-5A synthetase is precipitated and then incubated with ATP and poly(I).poly(C) to synthesize 2-5A oligomers.

2-5A Synthetase assay by immunoprecipitation

The 2-5A synthetase activity that is associated in immune complex preparations bound to protein A–Sepharose was assayed for its capacity to synthesize 2-5A. Crude extracts (100 μl, equivalent to that from 10^7 cells) from interferon-treated Daudi cells were first incubated (45 min, 4°C) with 2–5 μl of mouse serum before further incubation (3 h, 4°C) with protein A–Sepharose (100 μl) in buffer I.P. The immune complexes bound to protein A–Sepharose were washed batchwise and consecutively with three times 5 ml of buffer I.P. and twice in buffer A. Finally, the immune complexes bound to protein A–Sepharose were resuspended in a total reaction mixture (600 μl) containing: 20 mM Hepes pH 6.9, 50 mM KCl, 25 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol, 5 mM ATP, 10 mM creatine phosphate, 0.16 μg/ml of creatine kinase, 0.1 mg/ml of poly(I).poly(C) and 1 μl of [^{3}H]ATP (1 mCi/ml; 1.1–1.85 TBq/mmol). Incubation was for 17 h at 30°C and was terminated by heating at 90°C for 5 min. ^{3}H-labeled 2-5A was purified by DEAE–cellulose chromatography, as described previously (Buffet-Janvresse *et al.*, 1983). The results are either expressed in c.p.m. or in nanomoles of [^{3}H]AMP incorporated into 2-5A

The 2-5A synthetase assay using monoclonal antibodies 56/3 and 25/11 was carried out with 5 μl of ascitic fluid.

Fusion procedure

Splenocytes from the mouse producing anti-2-5A synthetase antibodies were fused in the presence of 50% polyethylene glycol 1500 (Roth, Karlsruhe, FRG) with X63/Ag8.653 myeloma cells. The fused cells were plated in 24-well plates (Limbro) and cultured as described (Laurent *et al.*, 1982). Culture supernatants (100–200 μl) were assayed for the production of anti-2-5A synthetase antibodies by the immunoprecipitation assay. Culture supernatants of X63 myeloma cells and sera from control and immune mice were used as negative and positive controls. Hybridoma cultures producing specific monoclonal antibodies were immediately subcloned by limiting dilution and injected into pristane-primed mice for production of ascitic fluid. Isotyping of monoclonal antibodies was determined by Ouchterlony's immunodiffusion test.

Electrophoretic transfer immunoblot analysis

Proteins (from 10^6 cells) were first subjected to electrophoresis in SDS–polyacrylamide slab gels before being electrophoretically transferred to 0.2 μM nitrocellulose sheets (Schleicher and Schüll, Dassel, FRG) in electrode buffer (20 mM Tris base, 150 mM glycine, 20% methanol, v/v), as described (Burnette, 1981). The electrophoretic blots were saturated with 5% (w/v) non-fat dry milk in PBS (Johnson *et al.*, 1984). They were then incubated in a sealed bag (overnight, 4°C) with rabbit anti-peptide B antibodies (at 1:100 dilution) in PBS containing 10% FCS and 0.2% Tween-20. The sheets were subsequently washed in PBS, PBS containing 5% Nonidet-40 and then re-saturated in PBS containing non-fat milk (5%). The washed sheets were then incubated (2 h, 4°C) in a sealed bag with a prepration of ^{125}I-labeled protein A (>30 mCi/mg). The sheets were removed from the bags and washed again, dried and autoradiographed (Kodak RP Royal, X-ray films) for 24–48 h.

Radioactive labeling of cells

Daudi cells in the presence of α-interferon (500 units/ml) were incubated for 6 h before labeling with 40 μCi/ml of [^{35}S]cysteine (22 TBq/mmol) in medium containing 1% of the normal level of cysteine (0.2 μg/ml). Cell extracts were prepared as above.

Preparation of different subcellular fractions

Cells were lysed in the homogenization buffer. The concentration of KCl was then increased to 75 mM and this suspension was centrifuged at 400 *g* for 10 min to collect the nuclei. For the preparation of nuclear extracts, the nuclei were first suspended in the homogenization buffer containing 5 mM $MgCl_2$ and then non-ionic detergent Triton X-100 was added to give a final concentration of 0.5%. This suspension was mixed gently for 2 min before 5-fold dilution in buffer A and centrifugation at 200 *g* for 10 min. This procedure was repeated twice to have a good preparation of nuclei without cytoplasmic contaminants (judged by phase microscopy). Nuclear extracts were prepared finally by suspension of nuclei in buffer I.P. These extracts were centrifuged at 10 000 *g* for 10 min, and the supernatant was collected and referred to as the nuclear fraction (NF). The post-nuclear supernatant fraction was first centrifuged at 2000 *g* for 10 min, and the supernatant was centrifuged at 12 000 *g* for 20 min to pellet mitochondria (P12 fraction). The post-mitochondrial supernatant (S12) fraction was centrifuged at 100 000 *g* for 2 h and the post-rough microsomal supernatant (S100) was collected. The tube containing the rough microsomal pellet (RMP) was rinsed in the homogenization buffer and then resuspended in buffer I.P. This suspension was left for 30 min at 4°C and centrifuged at 10 000 *g* for 10 min. The supernatant represented material extracted from RMP. The mitochondrial pellet was washed twice in the homogenization buffer by centrifugation (12 000 *g*, 10 min) and the pellet suspended in buffer I.P. The mitochondrial pellet is referred to as P12. All the procedures for the preparation of cell extracts were carried out at 4°C. Different cell fractions were stored at −80°C. S12, P12, S100, RMP and nuclear fractions had an equal final volume equivalent to 3×10^8 cells/ml. Purification of plasma membranes in the P12 fraction was carried out by centrifugation (22 000 *g* for 45 min) on a Percoll (Pharmacia) density gradient containing 150 mM NaCl.

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